AD_____

Award Number: W81XWH-04-1-0893

TITLE: High Throughput Screen to Identify Novel Drugs that Inhibit Prostate Cancer Metastasis

PRINCIPAL INVESTIGATOR: Irwin H. Gelman, Ph.D.

CONTRACTING ORGANIZATION: Roswell Park Cancer Institute
Buffalo, NY 14263

REPORT DATE: October 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2006	Annual	20 Sep 2005 – 19 Sep 2006

4. TITLE AND SUBTITLE

High Throughput Screen to Identify Novel Drugs that Inhibit Prostate Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0893

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Irwin H. Gelman, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Roswell Park Cancer Institute
Buffalo, NY 14263

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

We have proposed to developed indicator cell lines that would allow for the high throughput screening (HTS) for compounds that potentially inhibit prostate cancer (CaP) metastasis. The cell lines are based on stably expressing a construct containing the promoter of SSeCKS/ gravin/AKAP12- a metastasis-suppressor gene downregulated in CaP progression- linked to a green fluorescence protein (GFP), plus a control reporter, in metastatic CaP cells, and then screening for compounds that induce GFP. We also proposed to characterize the pathways controlling SSeCKS expression in CaP progression. UPDATE: Our data indicate that SSeCKS re-expression can be induced in CaP cell lines using inhibitors of histone deacetylation (TSA) but not by inhibitors of methylation (5-aza-C). We have now produced stable indicator C4-2 cells with GFP expression inducible by TSA and by retinoids. We have also characterized the cis- and trans-acting factors of the human SSeCKSα promoter required for transcriptional suppression in CaP cells.

15. SUBJECT TERMS
Drug discovery for prostate cancer using a cell-based assay. Prostate cancer, metastasis, drug discovery, SSeCKS/Gravin, HTS, tumor suppressor gene, gene regulation.

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

We have proposed to construct indicator prostate cancer (CaP) cell lines that could be used to identify novel drugs that could inhibit parameters of oncogenic and metastatic growth. The cell lines are based on the stable expression of the promoter from the SSeCKS/gravin/AKAP12 metastasis-suppressor fused to a green fluorescent protein (GFP) reporter, plus a control reporter plasmid. In a high throughput screen (HTS), compounds that induce GFP expression but have no major effect on the control reporter would be identified for further analysis as potential inhibitors of CaP progression. A second major aim of our study is to use the SSeCKS/gravin/AKAP12α promoter to characterize the signaling pathways as well as the *cis*- and *trans*-acting mechanisms leading to transcriptional downregulation in CaP cells. This analysis included a determination whether CaP-specific gene silencing involves hypermethylation of CpG islands in the SSeCKS promoters or changes in chromatin acetylation.

Body

I will first describe the progression we have made to produce and test the CaP indicator lines. Our first task (based on Task 1 in the Statement of Work) was to produce CaP lines stably expressing the human SSeCKS/gravinα promoter-GFP, plus a control reporter (described in Task 1 in the Statement of Work). The promoter was cloned into the pEGFP vector and in transient expression assays, this construct was shown to express high levels of green fluorescence in untransformed cells (murine NIH3T3 and human P69SV40T prostate epithelial) yet low GFP levels in LNCaP and C4-2 CaP cells. Instead of the originally intended control reporter (SEAP), we chose to use an RFP (red fluorescent protein) reporter (Clontech) that we fused to the TK promoter (Fig. 1). In transient assays, almost equal RFP expression levels were detected in all untransformed and CaP cell lines (data not shown). We then stably transfected P69, LNCaP and C4-2 cells with both plasmids, selected for neo^R colonies, and then FACS sorted pooled colonies for the desired phenotype: C4-2 and LNCaP cells were selected for low GFP, high RFP; P69 cell were selected for high GFP and RFP. 5 individual clones from each were expanded.

In order to fulfill the requirements of Task 4 (characterization of signaling pathways and transcription factors involved in SSeCKS transcriptional silencing in CaP cells), we then tested a panel of signal transduction inhibitors, differentiating agents and transcriptional deregulators for their ability to re-induced GFP and endogenous SSeCKS expression in the CaP cells while having minimal effect on RFP expression in either the CaP or P69 cells. Prior to this analysis, we first developed probes to detect changes in the levels of endogenous human SSeCKSα and β mRNA and protein isoforms. These included isoform-specific PCR primer sets (Fig. 2; was Fig. 3 in the 2005 update report), and from previous studies, polyclonal antibodies (Ab) that detected both α and β protein isoforms (3) as well as an Ab that detected only the α isoform (2).

Although data from other groups had shown cases where SSeCKS expression was suppressed by either promoter hypermethylation (1) or histone deacetylation (4), our data indicated that treatment of CaP cells with methylation inhibitors (5-aza-C or deoxy-5-aza-C) had no effect on transcript levels where the histone deacetylase inhibitor, TSA, derepressed SSeCKS transcript levels roughly 6- to 10-fold (although, still

significantly lower than untreated P69 cell levels). We then tested a panel of pathway inhibitory drugs to help define which pathways are responsible for SSeCKS downregulation in CaP and v-Src cells. Interestingly, endogenous SSeCKS was induced by TSA and by inhibitors of p38, PI3K (Fig. 3), Src or MEK (not shown), but only in the absence of serum. Given the previous finding that SSeCKS transcription is serum-responsive (5), our current data suggest that serum increases the background signal of SseCS mRNA, i.e.- masks the derepressive effects of the inhibitors. In contrast, the exogenous SSeCKS/GFP was not induced by inhibitors of Src, MEK, p38, PI3K, or by TSA, as shown by FACS analysis for GFP expression (Fig. 4). However, the differentiating agents, at-retinoic acid (Fig. 4) or calcitriol (not shown), did induce GFP expression. These FACS-based experiments were performed in the presence of serum. Indeed, Fig. 5 shows that TSA failed to induce endogenous SSeCKS expression in other human CaP lines grown in the presence of serum. Therefore, we are performing these same experiments in the absence of serum to determine if these inhibitory/differentiation compounds have greater derepressive effects on the exogenous promoter.

Continuing with Task 4 experiments, we addressed whether the endogenous human or mouse SSeCKS promoters could be induced by either synthetic testosterone (R1881) or by hypoxic conditions ($CoCl_2$). Fig. 6 shows that human SSeCKS can be induced by R1881, especially in the absence of serum, in keeping with previous data that it is an androgen-inducible gene (6). Fig. 6 also shows that in the absence of serum, SSeCKS is not induced under hypoxic conditions in both LNCaP NIH3T3 cells. This finding is in keeping with previous data showing that SSeCKS expression is not altered by hypoxia but is induced by the transition from hypoxia to normoxia (7).

In response to Task 3 of the Statement of Work, we analyzed the cis- and trans-acting factors that control SSeCKS promoter expression in CaP and 3T3/v-Src cells. We reasoned that common, if not overlapping control mechanisms and factors would be involved between the Src- and CaP-mediated SSeCKS downregulation. We previously identified minimal promoter fragment of -106 to +35 as encoding the CaP- and Src-responsive sequences in transient transfection, luciferase-reporter assays. Using EMSA and ChIP assays, we previously showed that this promoter fragment encodes an upstream E-box that binds USF1 and a downstream GC-box that binds both Sp1 and Sp3. Interestingly, even though both boxes are required for the CaP- and v-Src-associated SSeCKS downregulation, binding to the downstream Sp1/Sp3 box is increased roughly 4-fold in the transformed cells relative to the untransformed cell nuclear lysates.

In the current update, we show that the increased binding of Sp1/Sp3 to the GC-box of the SSeCKS promoter likely recruits histone deacetylases (HDAC) such as HDAC1, thereby converting what is an activation complex into a repression complex. Fig. 7 shows Src-transformed cells have relatively increased nuclear levels of Sp1 and Sp3 (~3-4 fold), and Fig. 9 shows that v-Src induces levels of nuclear HDAC1, but not HDAC2 or 3, roughly 3-fold. In keeping with the increased binding of Sp1 and Sp3 to the GC-box (EMSA assay) as shown in last year's update, we used chromatin immunoprecipitation (ChIP) analysis to show a similar increase in the association between Sp1/Sp3 and the endogenous SSeCKS promoters (Fig. 10). Interestingly, v-Src induced the enrichment of Sp1, but not Sp3, on the α promoter, whereas it induced the enrichment of Sp3, but not Sp1, on the β

promoter. The finding that TSA induces the re-expression of SSeCKS in CaP and Src-transformed cells implies that the suppression of the SSeCKS promoters involves increased recruitment of HDAC isoforms. Indeed, ChIP analysis shows that Src suppresses the association of acetylated histone-3 and –4 (markers of activated or "open" chromatin") on the α SSeCKS promoter, and that TSA induces their re-association (Fig. 8). The fact that neither Src nor TSA affects the association of Ac-H3 or –H4 with the β SSeCKS promoter suggests some sort of coordinated control directed by the α promoter, as has been suggested for the serum-response elements (5). We have had difficulty in showing increased binding of HDAC1 to the SSeCKS promoters by ChIP assay, likely due to the problems with the existing antibodies, and so we will attempt to show increased HDAC1 binding in the cancer cells by oligonucleotide pulldown using the SSeCKS GC-box domain. We also are attempting to knockdown HDAC1 (or HDAC2 or 3 as controls) by shRNA in order to show that the loss of HDAC1 in the cancer cells results in increased SSeCKS mRNA levels.

We used functional transient expression assays to show that Sp1 and Sp3 alone are inherent activators of the SSeCKS promoter, but when expressed with HDAC1, they become repressors. Specifically, 3T3 or 3T3/v-Src cells transfected with increasing amounts of Sp1 or Sp3 expressor plasmids resulted increased luciferase reporter expression (Fig. 11). Note that this activity in Sp3 required SUMOylation. Fig. 12 shows that transfection of increasing levels of an HDAC1-expressing plasmid alone causes the downregulation of the minimal α SSeCKS promoter activity, but roughly 3-fold more so in Src vs. 3T3 cells. Lastly, Fig. 13 shows that co-expression of increasing HDAC1 plus either Sp1 or Sp3 resulted in the sever downregulation of the α SSeCKS promoter in both 3T3 and 3T3/v-Src cells.

In sum, we have completed Task 1, made major strides in Task 3 and even have begun earlier than contemplated to start the experiments in Task 4, namely, to identify the transcription factors involved in CaP-associated SSeCKS downregulation. We have produced potentially usable indicator CaP cell lines required for Task 2, but induction of significant levels of GFP in these cells by inhibitors of what should be major oncogenic pathways has not been forthcoming. The fact that differentiation agents such as TSA or at-retinoic acid induce GFP expression in these lines leads us to believe we are on the correct track. We hope that in the coming year, we can use other human CaP cell lines such as DU145, LAPC-4 or CWR22rv11 in order to produce stable indicator lines with highly inducible GFP.

Based on our preliminary data showing that methylation does not play a major role in CaP-associated SSeCKS downregulation, but that histone deacetylation does, we have redirected much of our aims in Task 5 in order to characterize the deacetylases (HDAC) involved as well as the transacting factors that recruit the HDACs to the SSeCKS promoter region.

Key Research Accomplishments

-construction of SSeCKSα-GFP and TK-RFP reporter plasmids
-production of indicator CaP and P69 cell lines containing the SSeCKS and TK reporter plasmids.
-development of PCR and Ab-based reagents to detect SSeCKS mRNA and protein expression changes.
-demonstration that SSeCKS/gravin/AKAP12 derepression in CaP cells can be induced by TSA but not by 5-aza-C.

-demonstration that of the roughly 15-fold decrease in SSeCKS transcript levels in CaP vs. normal cells, 2-fold is controlled by decreases in transcript stability whereas the remaining portion is controlled by a 6- to 8-fold decrease in promoter activity levels.
-demonstration that the minimal CaP- and Src-responsive portion of the SSeCKS promoter is encoded between -106 and +35.
-identification of requirements for both upstream E- and downstream GC-box motifs for downregulation.
-demonstration that the E-box is occupied by USF1 (and not, for example, Myc) and that the GC-box is occupied by a combination of Sp1 and Sp3 (and not, MAZ).
-demonstration that the level of USF1 does not vary in CaP vs. normal cells, but that there is a relative 4-fold increase in Sp3:Sp1 in the transformed cells.
-ChIP assays to show *in vivo* association of SP1, Sp3 and USF with the SSeCKS promoters.
-functional assays showing that the SSeCKS promoters are downregulated by the recruitment of HDAC1 by Sp1/Sp3 in v-Src-transformed cells.
-development of first-generation C4-2 indicator lines with SSeCKS-GFP and showing inducible expression after TSA or at-retinoic acid treatment.

Reportable Outcomes

-Poster report, 2005 Oncogene Meeting, Frederick, MD, "Mapping of v-Src- and prostate cancer-responsive control sequences to the SSeCKS proximal promoter", Bu, Y. and Gelman, I.H., 6/21-24/2005.
-Poster report, 2006 Annual Meeting, American Assoc. of Cancer Research, Washington, DC, "Identification of v-Src- and prostate cancer-responsive sequences in the promoters of SSeCKS/Gravin/AKAP12, a metastasis-suppressor gene", Bu, Y. and Gelman, I.H., 4/1-5/2006.
-Development of CaP indicator cell lines and probes for SSeCKS isoform expression.

Conclusions

We have made major inroads in producing and characterizing the indicator lines we will use for the HTS drug screening, in characterizing the cis- and trans-factors controlling SSeCKS downregulation in CaP cells, and in elucidating the molecular mechanisms and pathways involved in SSeCKS transcriptional control.

References

1. **Choi, M. C., H. S. Jong, T. Y. Kim, S. H. Song, D. S. Lee, J. W. Lee, T. Y. Kim, N. K. Kim, and Y. J. Bang**. 2004. AKAP12/Gravin is inactivated by epigenetic mechanism in human gastric carcinoma and shows growth suppressor activity. Oncogene **23**:7095-7103.

2. **Gelman, I. H., E. Tombler, and J. Vargas, Jr.** 2000. A role for SSeCKS, a major protein kinase C substrate with tumor suppressor activity, in cytoskeletal architecture, formation of migratory processes, and cell migration during embryogenesis. Histochemical Journal **32**:13-26.

3. **Lin, X., E. Tombler, P. J. Nelson, M. Ross, and I. H. Gelman**. 1996. A novel *src*- and *ras*-suppressed protein kinase C substrate associated with cytoskeletal architecture. J.Biol.Chem. **271**:28,430-28,438.

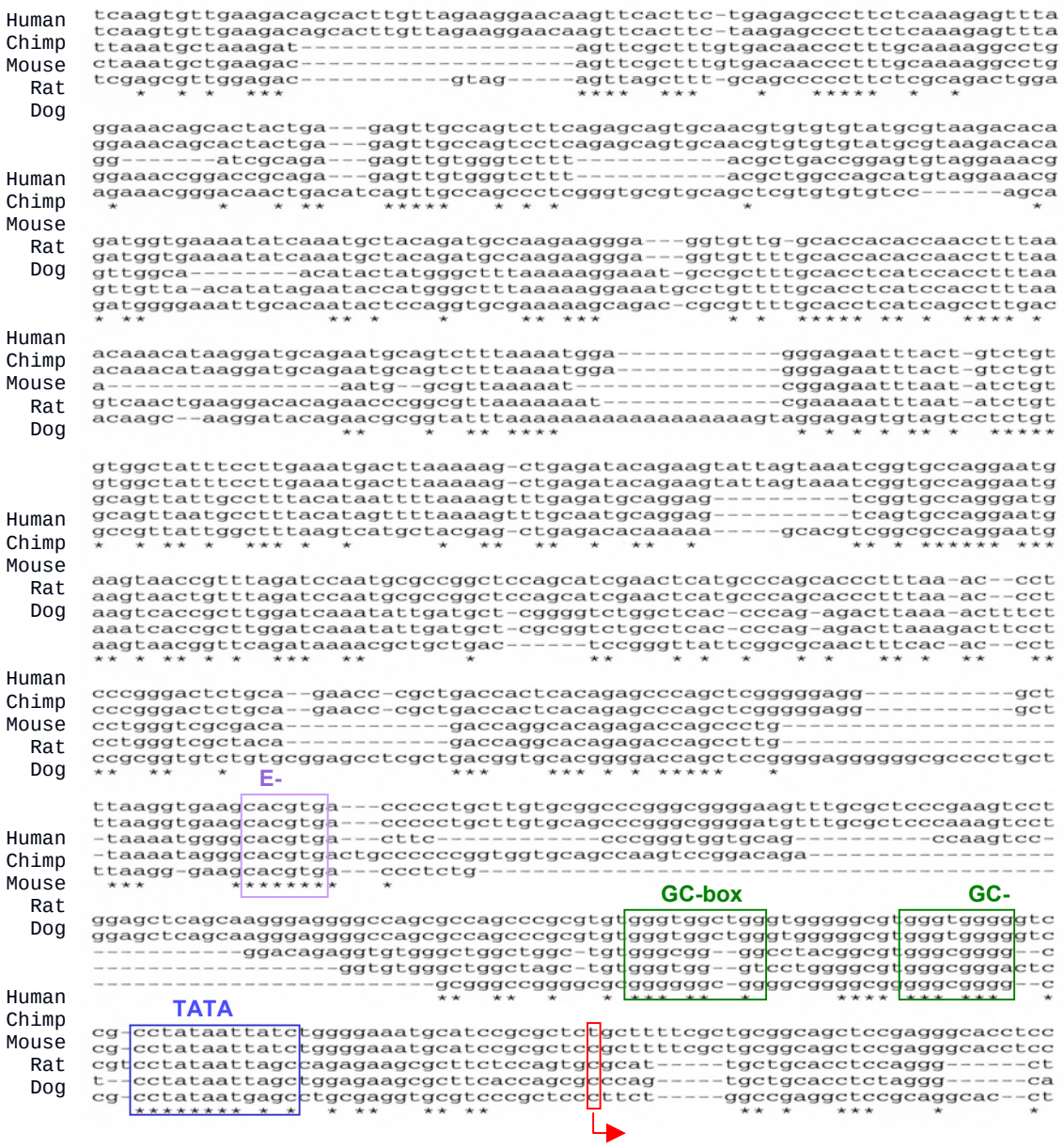
4. **Rombouts, K., T. Knittel, L. Machesky, F. Braet, A. Wielant, K. Hellemans, P. De Bleser, I. Gelman, G. Ramadori, and A. Geerts**. 2002. Actin filament formation, reorganization and migration are impaired in hepatic stellate cells under influence of trichostatin A, a histone deacetylase inhibitor. J.Hepatol. **37**:788-796.

5. **Streb, J.W., Kitchen, C.M., Gelman, I.H., Miano, J.M.** 2004. "Multiple promoters direct expression of the three AKAP12 isoforms with distinct subcellular and tissue distribution profiles" J. Biol. Chem. **279**:56014-56023.

6. **Nelson, PS; Clegg, N; Arnold, H; Ferguson, C; Bonham, M; White, J; Hood, L, & Lin, B**. 2002. The program of androgen-responsive genes in neoplastic prostate epithelium Proc. Natl. Acad. Sci. U. S. A, **99**:11890-11895.

7. **Lee, SW; Kim, WJ; Choi, YK; Song, HS; Son, MJ; Gelman, IH; Kim, YJ, & Kim, KW.** 2003. SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. Nature Medicine, **9**:900-906.

Appendices



Sequence alignment of SSeCKS α promoter regions in various mammalian species showing strong sequence conservation (*), especially in the retention and spacing of the E- and GC-box motifs just proximal to the transcriptional start site (red).

Supporting Data

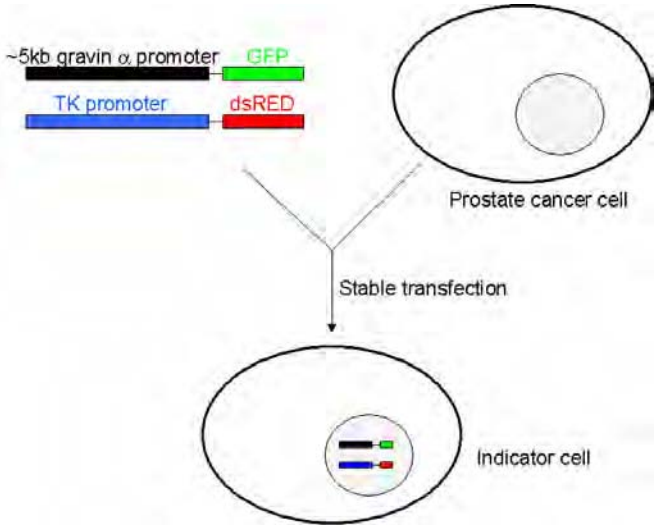


Figure 1. Production of indicator cells lines using the human α SSeCKS/Gravin/AKAP12 promoter.

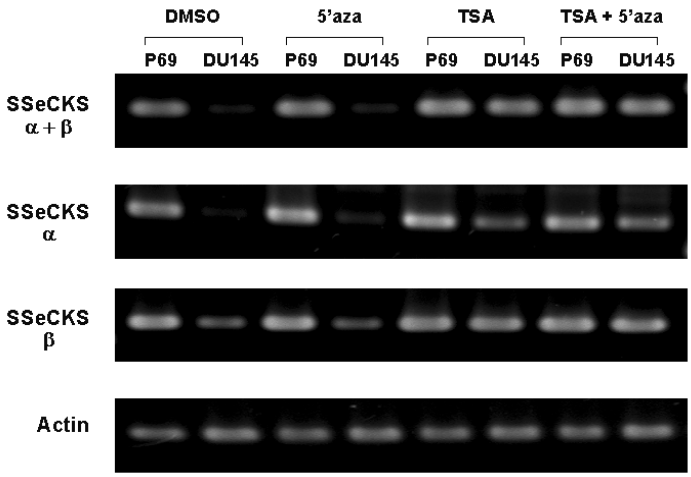


Figure 2. Semi-quantitative RT-PCR of SSeCKS (either the combined α/β, or α or β transcripts, versus actin as a control) showing that TSA, but not 5-azaC, derepresses SSeCKS expression in DU145 cells.

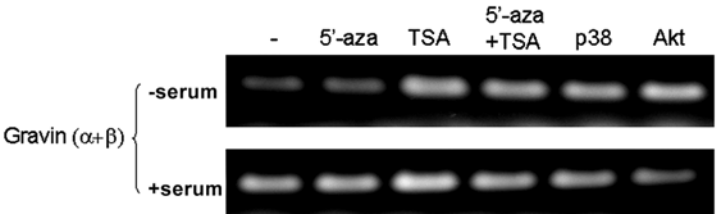


Figure 3. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β from C4-2 cells showing that TSA, the p38 and PI3K inhibitors, but not 5-azaC, derepresses SSeCKS expression only in the absence of serum.

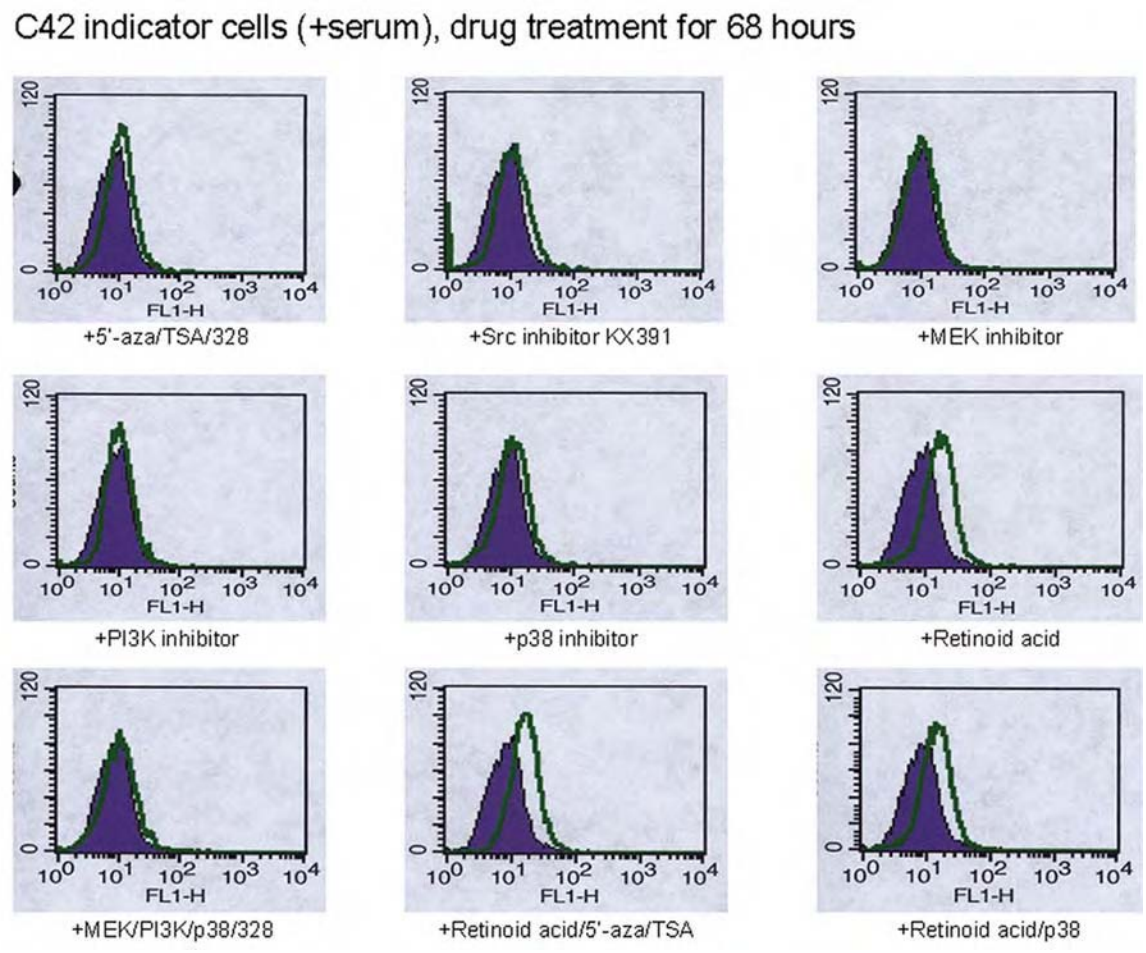


Figure 4. FACS cytometry analysis of GFP expression induced in the C4-2[SSeCKS/GFP] cells line by various signaling inhibitors or differentiation agents.

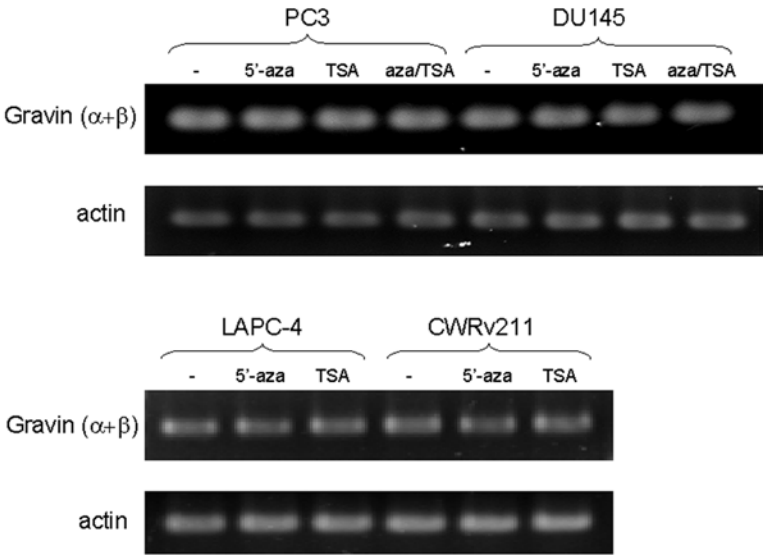
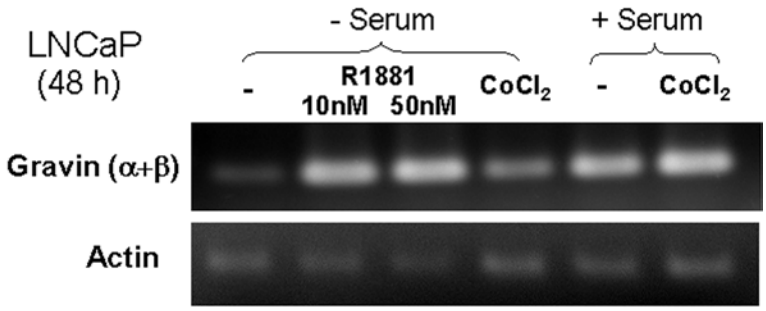


Figure 5. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β (vs. actin control) from CaP cells grown in the presence of serum, showing no SSeCKS derepression by TSA or 5-aza-C.



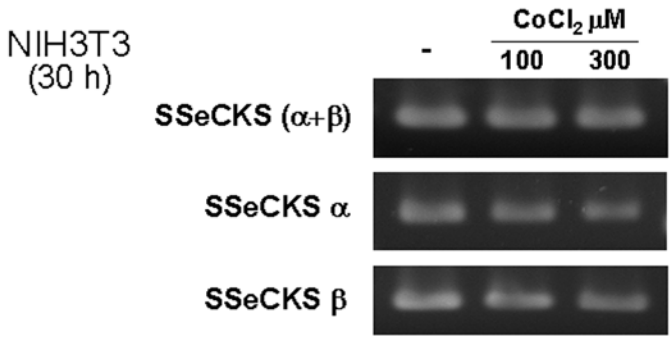


Figure 6. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β (vs. actin control) from LNCaP or NIH3T3 cells treated with either synthetic testosterone (R1881) or $CoCl_2$ (to induce hypoxic conditions).

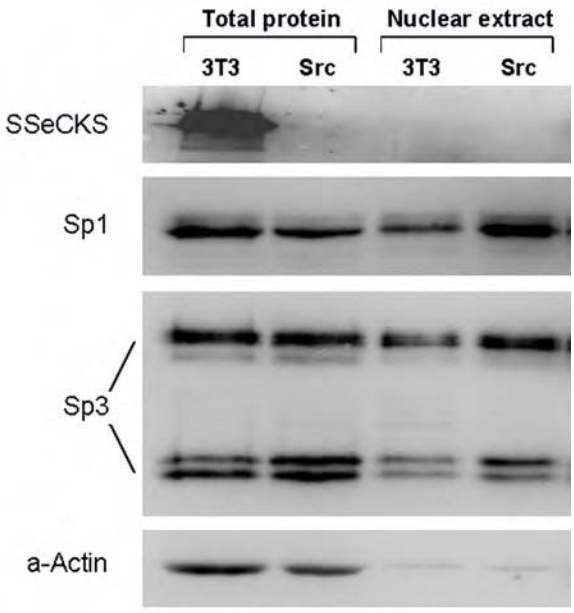


Figure 7. Immunoblotting of 3T3 or 3T3/v-Src nuclear or total cellular lysates for SSeCKS, Sp1, Sp3 and actin. Note the increased relative nuclear levels of Sp1 and Sp3 in Src-transformed cells.

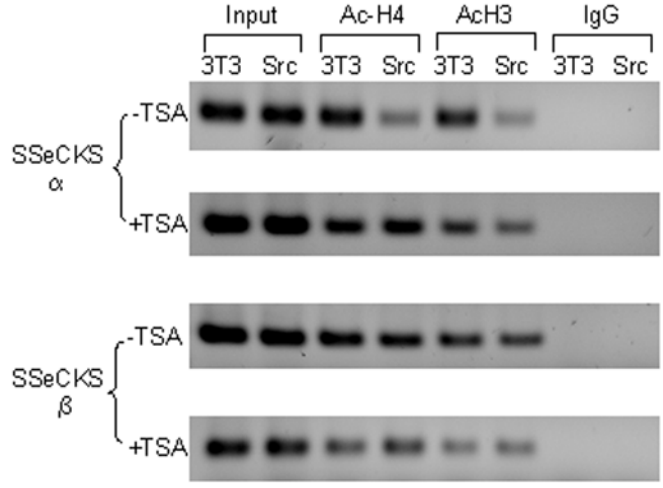


Figure 8. Chromatin immuno-precipitation of 3T3 or 3T3/v-Src nuclear lysates for acetylated histone 3 or 4 (vs. IgG control) followed by PCR amplification for either α or β SSeCKS. Note that Src suppresses the association of the Ac-histones with α but not β SSeCKS, and that TSA induces a re-association, implying that Src induces association of HDAC with the α promoter.

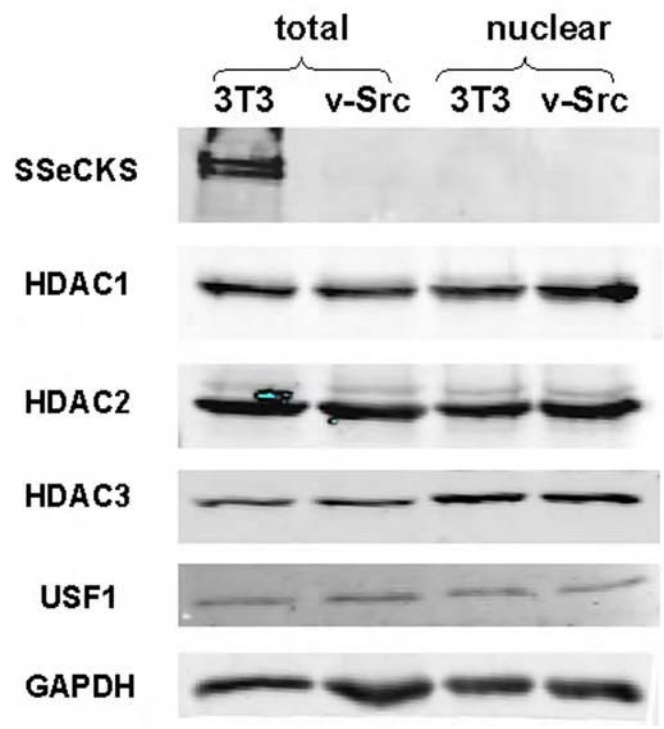


Figure 9. Immunoblotting of 3T3 or 3T3/v-Src nuclear or total cellular lysates for SSeCKS, HDAC1, 2, 3, or GADPH. Note the increased relative nuclear levels HDAC1 in Src-transformed cells.

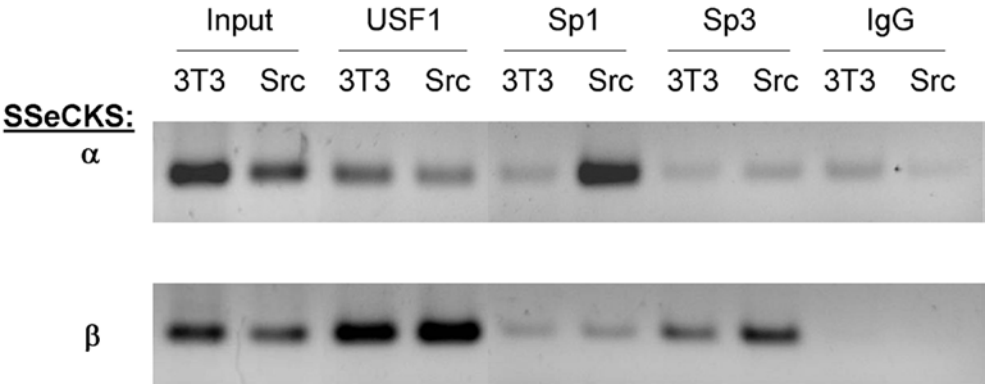


Figure 10. ChIP assay showing increased binding of Sp1 to the α promoter and increased Sp3 binding to the β promoter in 3T3/Src cells.

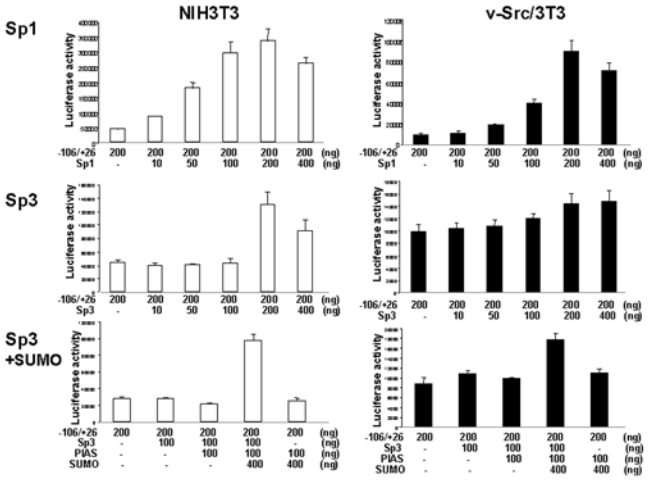


Figure 11. Transient transfection of 3T3 or 3T3/v-Src cells with the minimal αSSeCKS promoter/luciferase along with increasing amounts of Sp1 (top) or Sp3 (middle) expression plasmid, or Sp3 + a SUMO-expression plasmid (bottom).

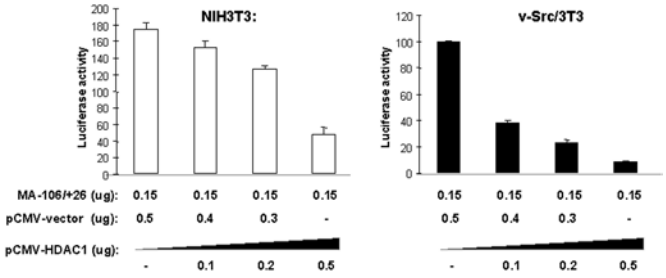


Figure 12. Similar experiment as in Fig. 10 except that the cells were transfected with increasing levels of an HDAC1-expressing plasmid. Note that HDAC1 induces greater relative suppression of the SSeCKS promoter in the Src cells.

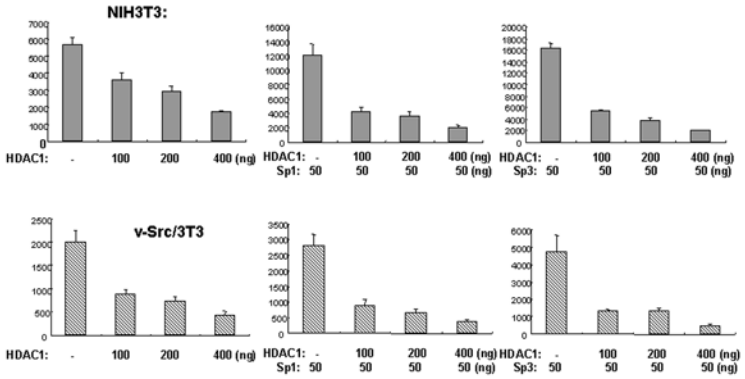


Figure 13. Similar experiment as in Fig. 10 except that the cells were transfected with constant levels of either Sp1- or Sp3-expressing plasmids plus increasing levels of an HDAC1-expressing plasmid.